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(54) **Title:** DRUG TARGET

(57) **Abstract:** Assays and screens for monitoring the expression of genes involved in the inflammatory response, such as the IFIT1, and for monitoring the activity of the products of such genes are useful in the identification of agents useful in the treatment of inflammatory disease.

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DRUG TARGET

FIELD OF THE INVENTION

[01] The present invention describes IFIT1 gene (Interferon-induced with tetratricopeptide repeats 1) as a molecular target for drugs to treat inflammatory disease and cancer and so relates to the fields of molecular biology, chemistry, pharmacology, and medicine.

BACKGROUND OF THE INVENTION

[02] Genomics approaches over the last decade have identified large numbers of novel genes of unknown function. A variety of techniques can be used to help define the function of such genes and their products. In one approach, the potential function of a novel gene may be inferred by sequence homology to genes or gene families with known function. In another approach, the pattern of gene expression is used as a guide to determining function. The most direct approaches to assigning gene function, however, involve the analysis of genetically modified cells or organisms in model systems, either by eliminating or by over-expressing the target gene or both.

[03] Useful model systems for the identification and validation of a novel gene as a potential target for the development of therapeutic agents are those that are relevant to disease pathophysiology. For example, endothelial cells are useful in disease models of inflammation. They respond to different inflammatory environments by differential expression of adhesion molecules and chemokines. Adhesion molecules, such as E- and P-selectin, ICAM-1 and VCAM-1 mediate the recruitment of leukocytes from the blood (Carlos and Harlan, 1994, *Blood 84*:2068). Leukocyte recruitment involves multiple steps, including rolling mediated by primary adhesion receptors such as E-, P- and L-selectin; chemoattractant mediated activation of secondary adhesion receptors, typically integrins; firm attachment mediated by integrin interactions with immunoglobulin gene family members, such as ICAM-1 and VCAM-1; and transendothelial migration (Butcher and Picker, 1996, *Science 272*:60; Springer, 1994, *Cell 76*:301).

[04] Fibroblasts play a key role in tissue remodeling that occurs during inflammation and wound repair. Fibroblasts secrete extracellular matrix proteins (e.g. collagens, laminin, fibronectin), matrix metalloproteinases (e.g. MMP-3) and tissue inhibitors of metalloproteinases (TIMPs), all of which are involved in building and/or degradation of connective tissue. Dis-regulation of expression of these proteins is responsible for tissue damage found in chronic inflammation, arthritis, cardiovascular diseases, pulmonary diseases and cancer. (Smeets et al., 2003, Ann Rheum Dis 62:635; Chevalier, 1997, Biomed Pharmacother 51:58; Chakraborti et al., 2003, Mol Cell Biochem 253:269). MMP-3

(transin or stromelysin) is an example of a matrix metalloproteinase elevated several fold in RA tissues (Hasty et al., 1990, Arthritis Rheum 33:388). MMP-3 is a secreted proteoglycanase closely related to collagenase (MMP1). It can degrade the major components of the extracellular matrix including proteoglycan, fibronectin, laminin, and type IV collagen, but not interstitial type I collagen (Sellers and Murphy, 1981, Int Rev Connect Tissue Res 9:151). .

[05] Therapeutic approaches that prevent leukocyte recruitment and at same time ameliorate inflammation-associated tissue damage have an enormous potential for treating a variety of diseases such as rheumatoid arthritis and psoriasis. Therapeutic strategies in clinical development that target the process of leukocyte recruitment include blocking ICAM-1, VCAM (through its ligand VLA-4), and the selectins, while the strategies that target tissue damage include development of matrix metalloproteinase inhibitors (Close, 2001, Ann Rheum Dis, 60 Suppl 3:iii6).

[06] Elevated levels of matrix metalloproteinases are also found in patients with aggressive forms of colorectal, breast, prostate and bladder cancers (Zuker et al., 1999, Ann N Y Acd Sci 878:212). Elevated levels of MMP-3 in particular seem to enhance susceptibility for development of breast cancer in animal models (Sternlicht et al., 2000, Oncogene 19:1102). Thus, genes that control expression of matrix metalloproteinases are also potential targets for development of anti-cancer drugs

[07] Expression of adhesion molecules (ICAM-1, VCAM) is transcriptionally regulated by cytokines such as TNF-alpha, IL-1beta, IFN-gamma, and IL-4 (Pober, 1998, *Pathol. Biol. 46*:159; Berg *et al.*, 1999, *Thromb. Haemost. Suppl.*, 624; Melrose *et al.*, 1998, *J. Immunol.* 161:2457; Yao *et al.*, 1996, *J. Exp. Med. 184*:81). Same cytokines, in particular IL-1beta, also regulate expression of matrix metalloproteinases (Hasty et al., 1990, Arthritis Rheum 33:388, MacNaul et al., 1990, J Biol Chem, 265:17238). TNF-alpha and IL-1beta activate the NFkB pathway, resulting in increased transcription of ICAM-1 , VCAM-1 and MMP-3 genes (Collins *et al.*, 1995, *Faseb J. 9*:899). IL-4 induces the expression of VCAM-1 on HUVEC cells through activation of Janus family kinases, including Jak1 and Jak3, and the STAT family member STAT6 (Nelms *et al.*, 1999, *Ann.. Rev. Immunol. 17*:701). On fibroblast however, IL-4 negatively regulates IL-1beta-stimulated MMP-3 expression (Prontera et al., 1996, Exp Cell Res 224:183). Stimulation with IFN-gamma results in activation of Jak1, Jak2, and STAT1. Activated STAT1 homodimers, or GAF, bind to GAS consensus sequences and induce expression of GAS-containing primary target genes (Decker *et al.*, 1997, *J. Interferon Cytokine Res. 17*:121). IFIT1 gene for example is induced by IFN-gamma. Some members of signaling pathways that control expression of adhesion molecules, chemokines and metalloproteinases have been identified including cytokine-induced pathways as well as various MAP kinase signaling cascades (Chakraborti et al.,

2003, Mol Cell Biochem 253:269). However, the precise mechanisms which control expression of these molecules are not well understood. There remains a need for the identification of other genes involved in these signaling pathways so that the products of such genes can be used as targets for the development of anti-inflammatory drugs.

[08] A variety of known drug compounds with specificity for a number of cellular targets affect the expression of cell surface adhesion molecules on cultured endothelial cells. These include aminosalicylates, corticosteroids, cyclooxygenase inhibitors, lipoxygenase inhibitors, and others (Volin *et al.*, 1999, *Arthritis Rheum. 42*:1927; Ray *et al.*, 1997, *Biochem. J. 328*:707; Cronstein *et al.*, 1992, *Proc. Natl. Acad. Sci. USA 89*:9991; Weber *et al.*, 1995, *Circulation 91*:1914; Pierce *et al.*, 1996, *J. Immunol. 156*:3961; Sakai, 1996, *Life Sci. 58*:2377; Menzel *et al.*, *Inflammation 23*:275; Lee *et al.*, 1996, *Immunol. Lett. 53*:10). In contrast, there has been little progress in developing successful MMP inhibitors. Several decades of research have yielded only one MMP inhibitor (batimastat) that has been approved for human therapeutic use (Greenwald, 1999, Ann N Y Acad Sci. 878:413). More recently, inhibitors of various kinases are showing some promise for blocking MMP expression (Chakraborti et al., 2003, Mol Cell Biochem 253:269). Thus, a method of screening for genes that influence the expression of both, cell adhesion molecules and matrix metalloproteinases could be useful for the identification and validation of new targets for therapeutic development. There remains a need for such targets and drugs that interact with them, in part due to the side effects of current anti-inflammatory therapeutics and lack of effective matrix metalloproteinase inhibitors. The present invention meets such needs by providing methods and reagents for screening compounds against target genes and their products to identify compounds useful for treatment of inflammatory diseases and cancer.

SUMMARY OF THE INVENTION

[09] The present invention provides assays and screens for identification of anti-inflammatory agents that modulate expression of pro-inflammatory proteins, including cell surface adhesion molecules, chemokines, matrix metalloproteinases, and HLA molecules. The interferon-induced with tetratricopeptide repeats 1 (IFIT1) protein is shown to modulate expression of pro-inflammatory proteins. The modulation of expression for certain proteins is revealed in the presence of cytokines, such as IL-1. In one embodiment of the invention, anti-inflammatory agents are identified by contacting cells expressing IFIT1; and measuring the alteration of expression of pro-inflammatory proteins, where agents that down-regulate expression of these proteins has anti-inflammatory activity. In another embodiment, anti-cancer agents are identified by contacting cells expressing IFIT1; and measuring the alteration of expression of cell surface adhesion molecules, chemokines, matrix metalloproteinases, and HLA molecules.

[10] In another embodiment of the invention, reagents are provided that are useful in the assays and screens of the invention. Such reagents include but are not limited to vectors for over-expressing or knocking-out IFIT1, cells containing such vectors, and DNA and antibody-based probes for detecting the genes and their RNA and protein products.

[11] In another embodiment of the invention, methods are provided for inhibiting inflammation by modulating activity of IFIT1. Modulating agents of interest include antibodies specific for the IFIT1 gene product, and pharmacologically active agents identified by the screening methods of the invention. In one embodiment, the agent decreases the expression of IFIT1 gene, or the activity of the IFIT1 gene product.

[12] In another aspect, the present invention provides an agent that modulates expression of IFIT1 gene, or modulates the activity of the IFIT1 gene product. In one embodiment, the agent decreases the expression of the gene or the activity of the gene product. In one embodiment, the agent is a gene therapy vector. In another embodiment, the agent is a protein or a small organic molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

[13] Figure 1. Effect of over-expression of TNF-alpha receptor (TNFRSF1A) and MEK2 genes on levels of ICAM-1, IL-8, HLA-DR and E-selectin on HUVEC cells. HUVEC cells were transduced with retroviral vector encoding TNFRSF1A and MEK2 genes and analyzed for ICAM-1, E-selectin, HLA-DR and IL-8 expression by ELISA. (A) Non-stimulated cells. (B) IFN-gamma-stimulated cells. (C) IL-1beta stimulated cells. Log transformed ratios of parameter values in gene-transduced relative to control cells [log10(IFIT1/Control)] are shown.

[14] Figure 2. Expression of IFIT11 gene upregulates ICAM-1 and IL-8 levels on HUVEC cells. HUVEC cells were transduced with retroviral vector encoding IFIT1 gene and analyzed for ICAM-1, E-selectin, HLA-DR and IL-8 expression by ELISA. (A) ICAM-1 levels on non-stimulated cells, (B) ICAM-1 levels on IFN-gamma-stimulated cells. (C) Log transformed ratios of ELISA OD values [log10(IFIT1/Control)] in IFN-gamma-stimulated cells are shown. Note that IFIT1 gene most strongly affects ICAM-1 expression. All ELISA OD values are from three independent experiments. Error bars indicate standard deviation from triplicate samples. Control cells were transduced with an "empty" retroviral vector, which does not encode any functional gene.

[15] Figure 3. Expression of IFIT1 up-regulates collagen I and MMP-3 on primary human fibroblasts. Cells were transduced with retroviral vector encoding IFIT1 gene and analyzed for expression of CD90, collagen I, HLA-DR, IL-8, IP-10, Mig, MMP-3, and MMP-1 by ELISA. (A) Non-stimulated cells. (B) IL-1beta-stimulated cells. Log transformed ratios of parameter values in IFIT1-transduced relative to control cells [$\log_{10}$(IFIT1/Control)] are shown. Note

specific up-regulation of MMP-3 and collagen I in IL-1beta-stimulated cells. Error bars indicate standard deviation from triplicate samples.

[16] Figure 4. HUVEC cells transduced with TNFRSF1A or MEK2 gene were treated with chemical compounds PD098059 and genistein. Where indicated cells were also stimulated with IL-1beta. Percent E-selectin levels relative to media control are shown. Data are average from triplicate experiments. Error bars indicate standard deviation.

Detailed Description of the Invention

[17] Assays and screens of the invention are useful for identification of anti-inflammatory agents, and anti-cancer agents. These agents are identified by functional assays that detect changes in the expression of molecules that create a pro-inflammatory environment, *e.g.* cell surface adhesion molecules, chemokines, matrix metalloproteinases, and HLA molecules. It is shown herein that the interferon-induced with tetratricopeptide repeats 1 (IFIT1) protein modulates expression of pro-inflammatory proteins by a cell, particularly in the presence of cytokines.

[18] Methods are provided for inhibiting inflammation by downregulating activity of IFIT1. Modulating agents of interest include antibodies specific for the IFIT1 gene product; and pharmacologically active agents identified by the screening methods of the invention. Methods are also provided for inhibiting growth of cancer cells by downregulating activity of IFIT1.

[19] The drug screening methods of the invention utilize cells, preferably primary human cells in culture, that express, or overexpress, a gene of interest. Screening is typically performed in the presence of exogenous agents that stimulate signaling pathways. Such exogenous agents, *e.g.* cytokines, have been found to activate cellular pathways that allow genes to reveal biological function only when other cellular signaling pathways are simultaneously active. Methods of screening genes are described, for example, in published U.S. Patent Application 10/236,558; and International Patent applications WO01/67103 and WO02/29097, each of which is herein incorporated by reference. Analysis of specific genes is provided in the examples.

[20] The preferred genes used in such assays are those whose function is not obvious from analysis of sequence or protein domain homology to known genes, either on protein or nucleotide level. These genes can be diverse in regard to their biochemical function and could include adaptor proteins, secreted factors, transcription factors, enzymes *etc.* The main characteristic of these genes is that they control or influence level of expression of other proteins (e.g. adhesion molecules, metalloproteinases) that are known to be important for inflammation, cancer or tissue remodeling (wound healing).

[21] In the present application, the IFIT1 is identified by an involvement in control of adhesion molecules, extracellular matrix proteins and matrix metalloproteinases involved in inflammation and cancer. IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1) gene is an example of a previously uncharacterized gene. The genetic sequence of IFIT1 may be obtained in public databases, e.g. Genbank accession number BC007091. Assays and screens for discovery of such a gene, and methods for treating inflammatory disease or cancer by modulating the expression of such a gene or by modulating the activity of such gene are described below

[22] IFIT1 gene expression is induced in cells treated with all three types of interferons (Chebath *et al.*, 1983, *Nucleic Acids Res.* 11:1213), and as such is found up-regulated in inflamed tissue where elevated levels of interferons are found, for example in systemic lupus erythematosus (SLE) (Ye et. al., 2003, Rheumatology 42:1155). The biological function of IFIT1 gene has not been previously reported. The only recognizable feature of the IFIT1 protein are tetratricopeptide repeats. Such domains are found on a range of proteins with diverse functions including anaphase promoting complex subunits, NADPH oxidase subunits, transcription factors, protein phosphatase 5 etc. (Das *et al.* (1998) EMBO J, 17:1192).

[23] As shown in the Examples, IFIT1 over-expression stimulates ICAM-1 and IL-8 expression on HUVEC cells and expression of collagen I and MMP-3 on fibroblasts. The effect of IFIT1 gene on collagen I and MMP-3 expression was seen only upon stimulation of fibroblasts with pro-inflammatory cytokine IL-1beta. This indicates a role for this gene in inflammation, in particular in modulation of genes involved in tissue remodeling.

[24] Thus, in one embodiment, the present invention provides a method for measuring the expression of the IFIT1 gene by detecting ICAM-1, IL-8, collagen I or MMP-3 expression. In another embodiment, the present invention provides a method for screening compounds to identify a compound that decreases IFIT1 expression or decreases the activity of the IFIT1 gene product, which method comprises contacting a cell that expresses IFIT1 with the compound, and determining whether ICAM-1 expression is decreased. In one embodiment, the cell is a cell that over-expresses IFIT1.

[25] Therapeutic and prophylactic treatment methods are provided for individuals suffering, or at risk of an inflammatory disorder, by administering an agent that modulates IFIT1 expression or the activity of the IFIT1 gene product by decreasing said expression or inhibiting said activity. In another embodiment, the present invention provides an agent that modulates IFIT1 expression or the activity of the IFIT1 gene product by decreasing said expression or inhibiting said activity, said agent being selected from the group consisting of a compound identified in the IFIT1 screen described above, an IFIT1 gene knock-out gene therapy vector, and an antibody that blocks the activity of the IFIT1 gene product.

[26] Screening methods generally involve conducting various types of assays to identify agents that modulate the expression or activity of IFIT1. Lead compounds identified during these screens can serve as the basis for the synthesis of more active analogs. Lead compounds and/or active analogs generated therefrom can be formulated into pharmaceutical compositions effective in treating neurological disorders such as stroke, epilepsy and neurodegenerative disorders.

[27] Expression of IFIT1 may utilize recombinant DNA technology using techniques well known in the art. Methods that are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. Alternatively, RNA capable of encoding the polypeptides of interest may be chemically synthesized.

[28] Typically, the coding sequence is placed under the control of a promoter that is functional in the desired host cell to produce relatively large quantities of the gene product. In one embodiment, the native IFIT1 promoter is used. In another embodiment, a heterologous promoter is used. An extremely wide variety of promoters are well-known, and can be used in the expression vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are also optionally included. Constructs that include one or more of these control sequences are termed "expression cassettes."

[29] In mammalian host cells, a number of viral-based expression systems may be used, including retrovirus, lentivirus, adenovirus, adeno associated virus, and the like. In cases where an adenovirus is used as an expression vector, the coding sequence of interest can be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing differentially expressed or pathway gene protein in infected hosts.

[30] Specific initiation signals may also be required for efficient translation of the genes. These signals include the ATG initiation codon and adjacent sequences. In cases where a complete gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the gene coding sequence is inserted, exogenous translational control signals must be provided. These exogenous translational control

signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.*

[31] Compound screening may be performed using a cell comprising an expression vector encoding IFIT1; or genetically modified to express IFIT1. A preferred cell for expression is a cell relevant to inflammation, e.g. fibroblasts, endothelial cells, including primary human endothelial cells, *e.g.* HUVEC; epithelial cells, smooth muscle cells, monocytes, T cells, *etc.* Preferred cells are fibroblasts, endothelial cells, and other cells relevant to inflammation.

[32] The IFIT1 coding sequences, as used herein, may include sequences that, by virtue of the degeneracy of the genetic code, are not identical in sequence to the disclosed nucleic acids; and may further include sequence variants of IFIT1. Variant polypeptides can include amino acid (aa) substitutions, additions or deletions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acids, such as to alter a glycosylation site, a phosphorylation site or an acetylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Variants can be designed so as to retain or have enhanced biological activity of a particular region of the protein (*e.g.*, a functional domain and/or, where the polypeptide is a member of a protein family, a region associated with a consensus sequence). Variants also include fragments of the polypeptides disclosed herein, particularly biologically active fragments and/or fragments corresponding to functional domains. Fragments of interest will typically be at least about 10 aa to at least about 15 aa in length, usually at least about 50 aa in length, and can be as long as 300 aa in length or longer, but will usually not exceed about 500 aa in length, where the fragment will have a contiguous stretch of amino acids that is identical to a polypeptide encoded by IFIT1, or a homolog thereof.

[33] Compound screening identifies agents that modulate inflammation. Of particular interest are screening assays for agents that have a low toxicity for human cells. A cell culture expressing, or capable of expressing, IFIT1 is contacted with a candidate agent, usually in the presence of a cytokine, where the cytokine is preferably IL-1β or a mimetic thereof. The activity of the agent may be determined by measuring alterations in the expression of inflammatory proteins, which include, without limitation, cytokines, adhesion molecules, metalloproteinases, and major histocompatibility antigens. Specific examples of suitable molecules include E-selectin; ICAM-1; Mig, IP-10; IL-8; MMP-3; MMP-1; HLA; and the like.

[34] The level of expression or activity can be compared to a baseline value. The baseline value can be a value for a control sample or a statistical value that is representative of a control sample. Expression levels can also be determined for cells that do not express IFIT1; or that have not been contacted with an activating cytokine as a negative control. Such cells generally are otherwise substantially genetically the same as the test cells.

[35] Various controls can be conducted to ensure that an observed activity is authentic including running parallel reactions with cells that lack the expression construct or by not contacting a cell harboring the expression construct with test compound. Compounds can also be further validated as described below.

[36] The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function of IFIT1. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

[37] Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[38] Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs. Test agents can be obtained from libraries, such as natural product libraries or combinatorial libraries, for example. A number of different types of combinatorial libraries and methods for preparing such libraries

have been described, including for example, PCT publications WO 93/06121, WO 95/12608, WO 95/35503, WO 94/08051 and WO 95/30642, each of which is incorporated herein by reference.

[39] A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, *e.g.* albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the biological activity. Incubations are performed at any suitable temperature, typically physiological temperatures. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening.

[40] Compounds that are initially identified by any of the foregoing screening methods can be further tested to validate the apparent activity. The basic format of such methods involves administering a lead compound identified during an initial screen to an animal that serves as a model for humans and then determining if inflammation is affected in an *in vivo* setting. The animal models utilized in validation studies generally are mammals. Specific examples of suitable animals include, but are not limited to, primates, mice, and rats.

[41] Active test agents identified by the screening methods described herein that modulate inflammation can serve as lead compounds for the synthesis of analog compounds. Typically, the analog compounds are synthesized to have an electronic configuration and a molecular conformation similar to that of the lead compound. Identification of analog compounds can be performed through use of techniques such as self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available. See, e.g., Rein et al., (1989) Computer-Assisted Modeling of Receptor-Ligand Interactions (Alan Liss, New York).

[42] Once analogs have been prepared, they can be screened using the methods disclosed herein to identify those analogs that exhibit an increased ability to modulate inflammation. The cycle of screening, synthesizing analogs and re-screening can be repeated multiple times.

PHARMACEUTICAL COMPOSITIONS

[43] Compounds identified by the screening methods described above and analogs thereof can serve as the active ingredient in pharmaceutical compositions formulated for the treatment of various inflammatory disorders. The compositions can also include various other agents to enhance delivery and efficacy. The compositions can also include various agents to enhance delivery and stability of the active ingredients.

[44] Thus, for example, the compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers of diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can include other carriers, adjuvants, or non-toxic, nontherapeutic, nonimmunogenic stabilizers, excipients and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents, wetting agents and detergents.

[45] The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. When the pharmaceutical composition includes a polypeptide, the polypeptide can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (*e.g.,* increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or complexing agents include sulfate, gluconate, citrate and phosphate. The polypeptides of a composition can also be complexed with molecules that enhance their *in vivo* attributes. Such molecules include, for example, carbohydrates, polyamines, amino acids, other peptides, ions (*e.g.,* sodium, potassium, calcium, magnesium, manganese), and lipids.

[46] Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990).

[47] The pharmaceutical compositions can be administered for prophylactic and/or therapeutic treatments. Toxicity and therapeutic efficacy of the active ingredient can be determined according to standard pharmaceutical procedures in cell cultures and/or experimental animals, including, for example, determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred.

[48] The data obtained from cell culture and/or animal studies can be used in formulating a range of dosages for humans. The dosage of the active ingredient typically lines within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage

can vary within this range depending upon the dosage form employed and the route of administration utilized.

[49] The pharmaceutical compositions described herein can be administered in a variety of different ways. Examples include administering a composition containing a pharmaceutically acceptable carrier via oral, intranasal, rectal, topical, intraperitoneal, intravenous, intramuscular, subcutaneous, subdermal, transdermal, intrathecal, and intracranial methods.

[50] The components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (e.g., at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade). Moreover, compositions intended for in vivo use are usually sterile. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process. Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

THERAPEUTIC/PROPHYLACTIC TREATMENT METHODS

[51] Agents that modulate inflammation through IFIT1 provide a point of therapeutic or prophylactic intervention. Numerous agents are useful in modulating this activity, including agents that directly modulate expression, *e.g.* expression vectors, antisense specific for IFIT1; and agents that act on the protein, *e.g.* specific antibodies and analogs thereof, small organic molecules that block activity, *etc.* Agents may be administered to patients suffering from an inflammatory disease.

[52] Inflammation is a defense reaction caused by tissue damage or injury, characterized by redness, heat, swelling, and pain. The primary objective of inflammation is to localize and eradicate the irritant and repair the surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process. The inflammatory response involves three major stages: first, dilation of capillaries to increase blood flow; second, microvascular structural changes and escape of plasma proteins from the bloodstream; and third, leukocyte transmigration through endothelium and accumulation at the site of injury.

[53] The accumulation of leukocytes in inflamed tissue results from adhesive interactions between leukocytes and endothelial cells within the microcirculation. These adhesive interactions and the excessive filtration of fluid and protein that accompanies an inflammatory response are largely confined to one region of the microvasculature: postcapillary venules. The nature and magnitude of the leukocyte-endothelial cell adhesive interactions that take place within postcapillary venules are determined by a variety of

factors, including expression of adhesion molecules on leukocytes and/or endothelial cells, products of leukocyte (superoxide) and endothelial cell (nitric oxide) activation, and the physical forces generated by the movement of blood along the vessel wall.

[54] Inflammatory diseases are caused by abnormal immune responses such as immune system turning on itself to attack the very tissues it has evolved to protect. Seemingly unrelated disorders such as asthma, multiple sclerosis, inflammatory bowel diseases and rheumatoid arthritis all have common inflammatory elements that underlie the disease process. Examples of inflammatory diseases include: rheumatoid arthritis; multiple sclerosis; inflammatory bowel disease, psoriasis; chronic obstructive pulmonary disease; asthma; among others.

EXPERIMENTAL

[55] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[56] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[57] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

EXAMPLE 1

[58] The methods of the invention are exemplified here for two genes, TNFRSF1A (TNF-alpha receptor type I), and a kinase MEK2 (expressed here as a constitutively active mutant, Mansour et al., 1994, Science 265:966). Over-expression of TNFRSF1A increases E-selectin, ICAM-1 and IL-8 levels in non-stimulated and IFN-gamma stimulated HUVEC cells (Figure 1A & 1B). An additional effect observed for TNFRSF1A in IFN-gamma-stimulated cells only is strong down-regulation of HLA-DR expression. In contrast, MEK2 gene is inactive in non-stimulated and IFN-gamma stimulated HUVEC cells, but it strongly down-regulates E-selectin and IL-8 in IL-1beta-stimulated cells (Figure 1C). Conversely, TNFRSF1A does not show activity in IL-1beta-stimulated cells (Figure 1C). Thus, the assays described here and which utilizes endothelial cells, allow identification of genes that control expression of adhesion molecules and chemokines relevant for inflammation, and thus such genes could serve as potential targets for drug development. Indeed, soluble form of the TNFRSF1A (Enbrel) is clinically used as therapeutic agent for rheumatoid arthritis.

EXAMPLE 2

[59] This example demonstrates the method of the invention for determining the biological function of a gene by over-expression of the gene in a cell and measurement of a variety of indicators of gene function, preferably in a number of different cellular environments (for example, exposure to cytokines). As demonstrated in this Example, over-expression of IFIT1 gene modulates expression of ICAM-1, and IL-8 on endothelial cells, and MMP-3 and collagen I on fibroblasts. It is important to note that the effect of IFIT1 gene on MMP-3 and collagen I expression is observed only upon stimulation of cells with IL-1beta.

[60] Primary human umbilical vein endothelial cells (HUVEC) and primary human dermal fibroblasts were used in the method. Other primary cell types might be used, for example primary microvascular endothelial cells, aortic or arteriolar endothelial cells, bronchial epithelial cells, smooth muscle cells, monocytes, T cells etc.

[61] Exponentially growing cells were transduced with a retroviral vectors encoding IFIT1 gene. Other gene delivery methods could be used as well. These gene delivery methods include, but are not limited to, methods employing other viral vectors (AAV, Adeno, Lenti, SV40, HSV, and the like), electroporation, biochemical methods (cationic lipids, calcium phosphate, DEAE, dextran, and the like), particle bombardment ("gene gun"), and the like.

[62] A marker gene is incorporated in the vector that allows monitoring of expression. A suitable retroviral vector is derived from the MoMLV-based pFB vector (Stratagene). Test genes are inserted downstream of the MoMLV LTR. The marker gene can be the truncated form of the human nerve growth factor receptor (NGFR) (Mavilio, 1994, *Blood* 83:1988) separated from the test gene by an independent ribosomal entry site sequence (IRES). The

IRES is 100 bp fragment from human eIF4G IRES sequence (Gan, W. Biol. Chem. 273:5006, 1988).

[63] Retroviral vector plasmid DNA is transfected into AmphoPack-293 cells (Clonetech) by the modified calcium phosphate method according to the manufacturer's protocol (MBS transfection kit, Stratagene). Cell supernatants are harvested 48 hours post-transfection, filtered to remove cell debris (0.45 micrometer), and transferred onto exponentially growing HUVEC cells or fibroblasts. DEAE dextran (conc 10 microgram/ml) is added to facilitate vector transduction. After a 5-8 hour incubation, the viral supernatant is removed and cells cultured for an additional 40 hours. Gene transfer efficiency can be determined by FACS using NGFR-specific monoclonal antibodies, and is typically ≥70%. Transduced cells are re-plated into 96-well plates, and cultured to confluence for analysis of expression of adhesion and other molecules of interest. In some experiments, confluent transduced or control cells are treated with IFN-gamma (100 ng/ml) or IL-1beta (1 ng/ml) for 24 hours. After 24 hours, cultures are washed and evaluated for the cell surface expression of ICAM-1, E-selectin , IL-8, HLA-DR on HUVEC cells, and HLA-DR, IL-8,MIG, CD90, collagen I, MMP-3, MMP-1 and IP-10 on fibroblasts. All protein measurements were performed by ELISA (Melrose, 1998, *supra*). In some tests, a change in ICAM-1 expression was confirmed by FACS analysis. Average ELISA OD values from triplicate samples are used to generate log-transformed ratios between gene-transduced and control cells (transduced with the "empty" vector, which only encodes the NGFr marker).

[64] The results in Figure 2 demonstrate that IFIT1 gene up-regulates expression of ICAM-1 and IL-8 on non-stimulated HUVEC cells. In addition, IFIT1-1 gene further increases the levels of ICAM induced by stimulation of cells with interferon-gamma (Figure 2B). The expression of antigen-presenting molecule HLA-DR, and adhesion molecule E-selectin was not affected by the IFIT1 gene (Figure 2C), indicating that the observed effect on the ICAM-1 and IL-8 genes is caused by a specific mechanism and is not due to general effect of IFIT1 on cellular gene expression. These data indicate that IFIT1 gene could serve as a target for inhibition of ICAM-1 and IL-8 expression on endothelial cells and thus modulation of the inflammatory response.

[65] The results in Figure 3 demonstrate that IFIT1 gene up-regulates expression of collagen I and MMP-3 on IL-1beta-stimulated fibroblasts. The expression of HLA-DR, IL-8, MIG, CD90, MMP-1 and IP-10 was not affected by the IFIT1 gene (Figure 3), indicating that the observed effect on the collagen I and MMP-3 genes is caused by a specific mechanism and is not due to general effect of IFIT1 on cellular gene expression. These data indicate that IFIT1 gene could serve as a target for inhibition of collagen I and MMP-3 expression on fibroblast cells, and thus for modulation of the inflammatory response, tissue remodeling or cancer progression.

[66] Protein markers (e.g. E-selectin), which were up-regulated on cells upon over-expression of the gene of interest can be used to set-up screening assays for inhibitors of over-expressed genes. For example, cells over-expressing TNFRSF1A gene show elevated levels of E-selectin (Figure 1A & 1B) while cell over-expressing MEK2 and stimulated with IL-1beta show reduced levels of E-selectin compared to control (Figure 1C). Thus, inhibition of the over-expressed gene activity would result in reduced E-selectin levels in the case of TNFRSF1A, and increased levels in the case of MEK2 (inhibiting activity of an inhibitory gene gives a positive response).

[67] HUVEC cells were transduced with retroviral vectors expressing TNFRSF1A and MEK2 genes as described above. HUVEC cells were obtained from Clonetics and cultured in EGM-2 medium containing supplements provided by the manufacturer and 2% heat inactivated fetal bovine serum (Hyclone, Logan, Utah) and subcultured with 0.05%trypsin-0.53mM EDTA (Mediatech, Herndon, Virginia) as described by the manufacturer. Experiments were performed by culturing cells in microtiter plates (Falcon; BD Biosciences), and where indicated, in the presence of cytokine IL-1beta at 1 ng/ml. Drugs PD098059 (3.7 micromolar) and genistein (10 micromolar) were added 1 hr before stimulation and were present during the whole 24 hr stimulation period. E-selectin levels were measured by ELISA using mouse anti-human E-selectin (clone ENA1, mIgG1) from HyCult Biotechnology (Uden, The Netherlands).

[68] PD098059 is a known MEK inhibitor and as such shows specific inhibition of the over-expressed MEK2 gene. Since MEK2 gene reduces E-selectin levels in IL-1beta stimulated cells, blocking MEK2 actually results in elevation of the E-selectin levels. This is shown in Figure 4, where treatment of MEK2-expressing cells with PD09059 increases E-selectin levels 3-fold compared to MEK2-expressing cells treated with DMSO as buffer control. Conversely, TNFRSF1A gene up-regulates E-selectin, and therefore blocking TNFRSF1A gene activity will result in lower E-selectin levels. This is shown in Figure 4, where treatment of TNFRSF1A-expressing cells with genistein reduces E-selectin levels approximately 2-fold compared to TNFRSF1A-expressing cells treated with DMSO as buffer control. The effect of these drugs is gene-specific, PD09059 had no effect on TNFRSF1A gene and genistein had no effect on MEK2 gene.

[69] A screen for inhibitors of the IFIT1 gene would include over-expression of the IFIT1 gene in HUVEC cells, fibroblasts or other suitable cell type, contacting those cells with chemical compounds at various concentrations for a specified length of time (e.g. 24 hours), stimulation of these cells with an appropriate external agent (e.g. IL-1beta), and subsequent measurement of appropriate markers (e.g. ICAM-1, IL-8, Collagen I, MMP-3). The drugs of interests might either increase or decrease the marker level. These and other aspects of the

invention will be apparent to those of skill in the art to which this invention pertains. All references cited in this specification are incorporated herein by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method to identify a candidate agent having anti-inflammatory activity, the method comprising:

contacting a cell expressing IFIT1 with said candidate agent;

measuring the expression of one ore more inflammation associated genes regulated by IFIT1,

wherein a down regulation of expression of said inflammation associated genes is indicative of anti-inflammatory activity by said candidate agent.

2. The method according to Claim 1, wherein said cell is a primary human cell.

3. The method according to Claim 2, wherein said cell is cultured in the presence of one or more cytokines.

4. The method according to Claim 3, wherein said one or more cytokines comprises IL-1beta.

5. The method according to Claim 2, wherein said one ore more inflammation associated genes comprises chemokines, metalloproteinases, extracellular matrix proteins, and major histocompatibility antigens.

6. The method according to Claim 5, wherein said one ore more inflammation associated genes comprises E-selectin, ICAM-1, Mig, IP-10 and IL-8, collagen I, MMP-1, MMP-3, and HLA .

7. A reagent useful in the method of Claim 1 consisting of a vector for over-expressing or knocking-out IFIT1 gene, cells containing such vectors, and DNA and antibody-based probes for detecting the genes and their RNA and protein products.

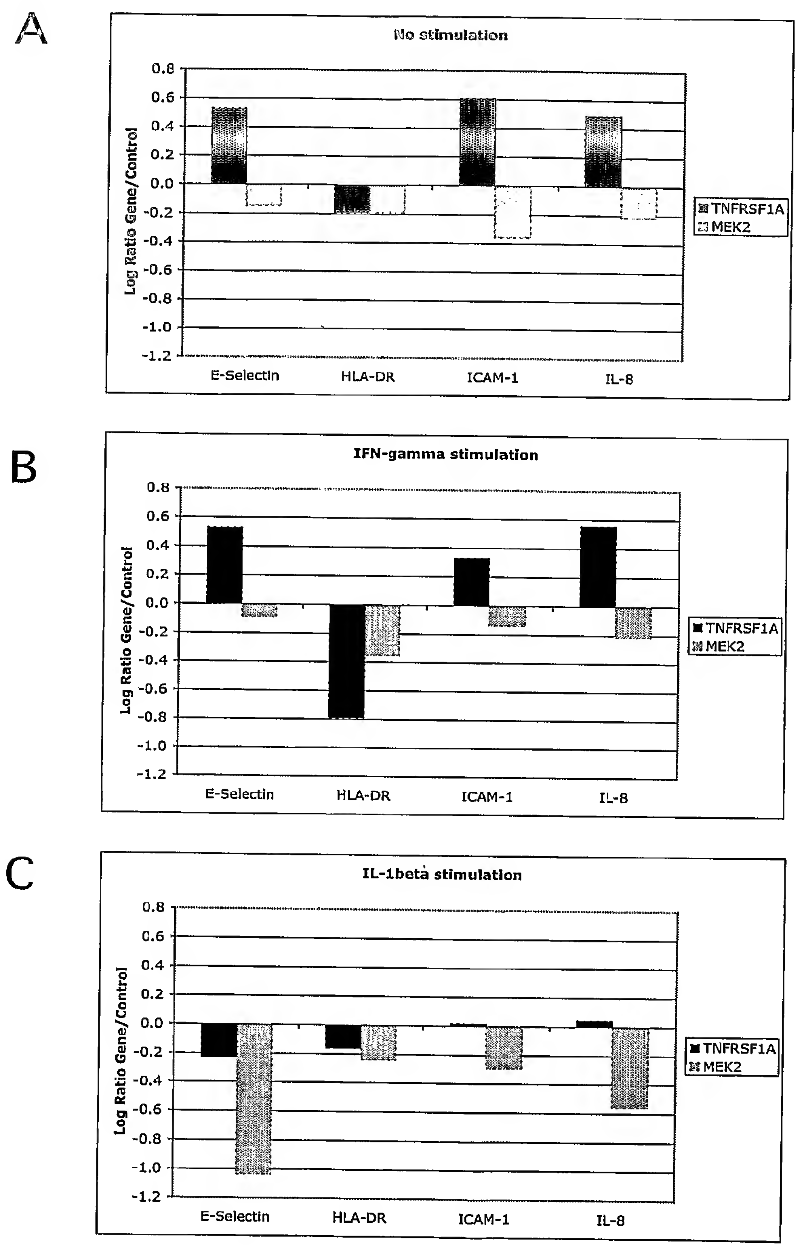
8. A method for treating an inflammatory disease, which method comprises administering an agent that modulates expression of IFIT1 gene.

9. The method of Claim 3, wherein the agent decreases the expression of the gene or the activity of the gene product.

10. An agent that modulates expression of the IFIT1 gene or modulates the activity of a product of such a gene.

11. The agent of Claim 10 that decreases the expression of the gene or the activity of the gene product.

12. The agent of Claim 11 that is selected from the group consisting of a gene therapy vector, a protein, or a small organic molecule.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0.6
0.4
0.2
0.0
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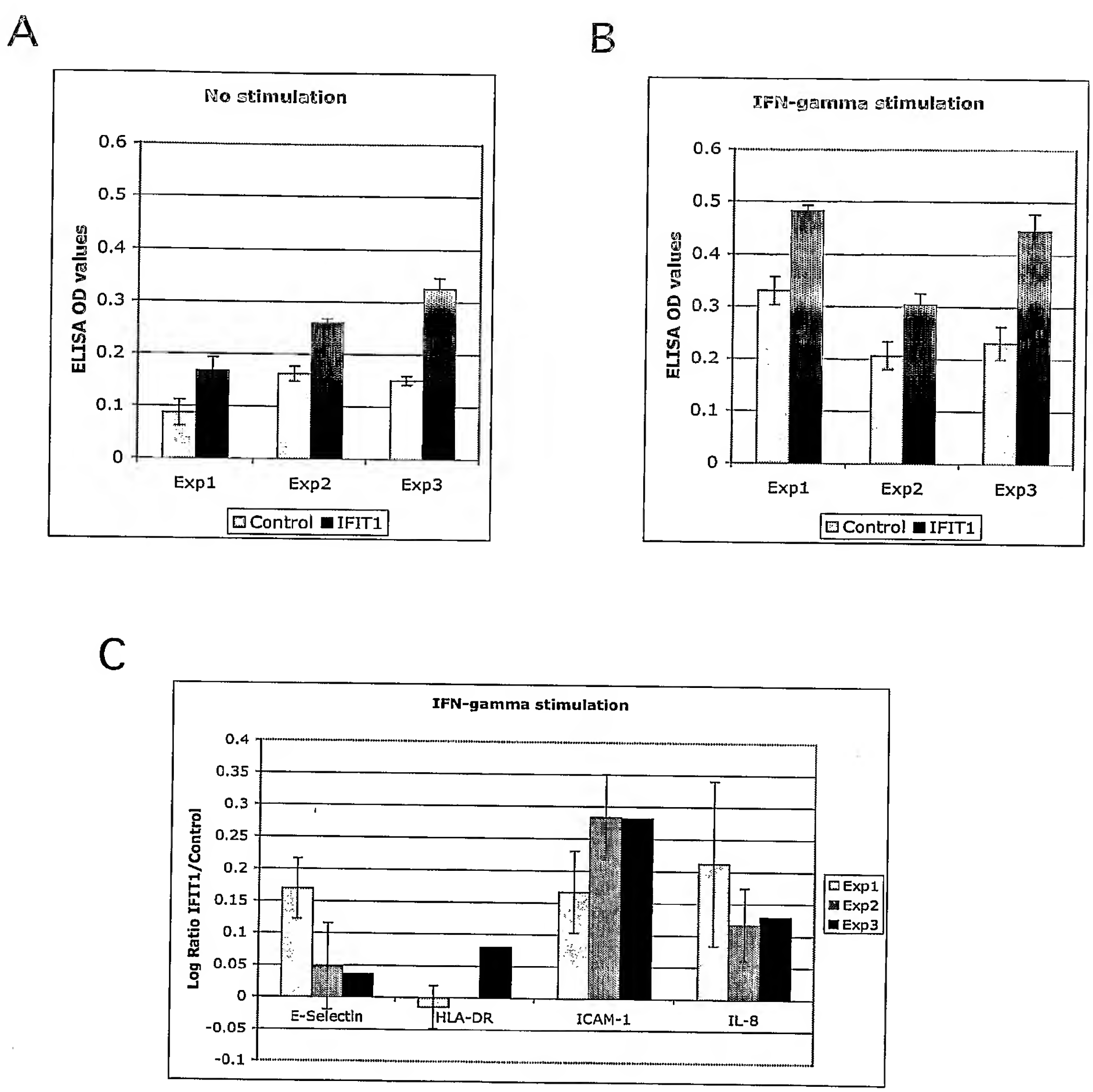
-0.8
-1.0
-1.2
E-Selectin
HLA-DR
ICAM-1
IL-8
TNFRSF1A
MEK2
B
IFN-gamma stimulation
C
IL-1beta stimulation

Figure 1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0.4
0.3
0.2
0.1
0
Exp1
Exp2
Exp3
Control
IFIT1
B
IFN-gamma stimulation
ELISA OD values
0.6
0.5
0.4
0.3
0.2
0.1
0
Exp1
Exp2
Exp3
Control
IFIT1
C
IFN-gamma stimulation
Log Ratio IFIT1/Control
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
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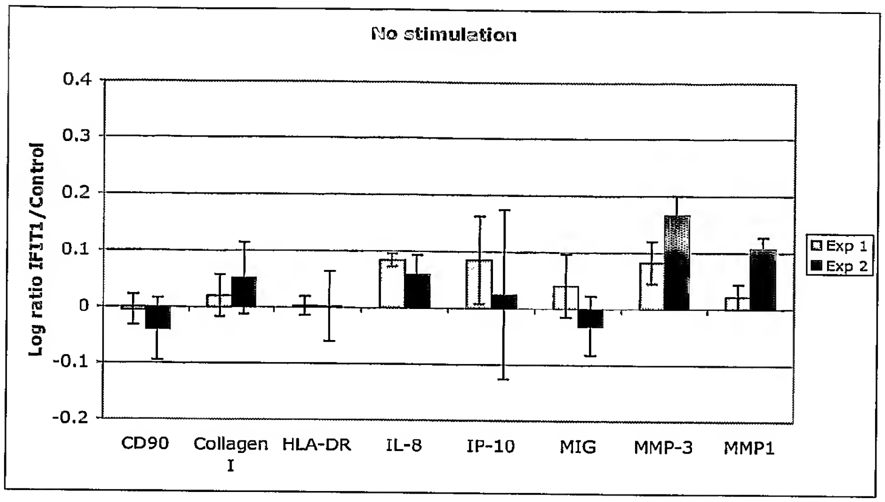
-0.1
E-Selectin
HLA-DR
ICAM-1
IL-8
Exp1
Exp2
Exp3

Figure 2

A


No stimulation
Log ratio IFIT1/Control
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0.3
0.2
0.1
0
-0.1
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CD90
Collagen I
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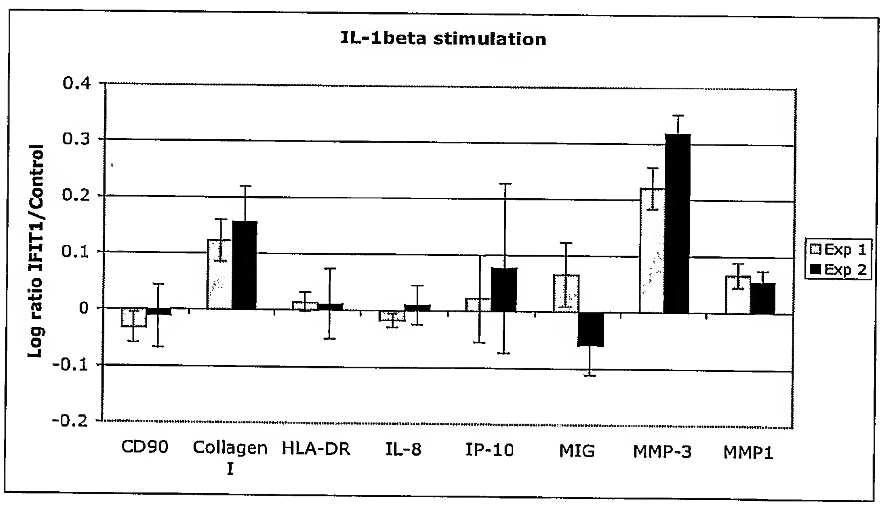
IL-8
IP-10
MIG
MMP-3
MMP1
Exp 1
Exp 2

B


IL-1beta stimulation
Log ratio IFIT1/Control
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0.3
0.2
0.1
0
-0.1
-0.2
CD90
Collagen I
HLA-DR
IL-8
IP-10
MIG
MMP-3
MMP1
Exp 1
Exp 2

Figure 3

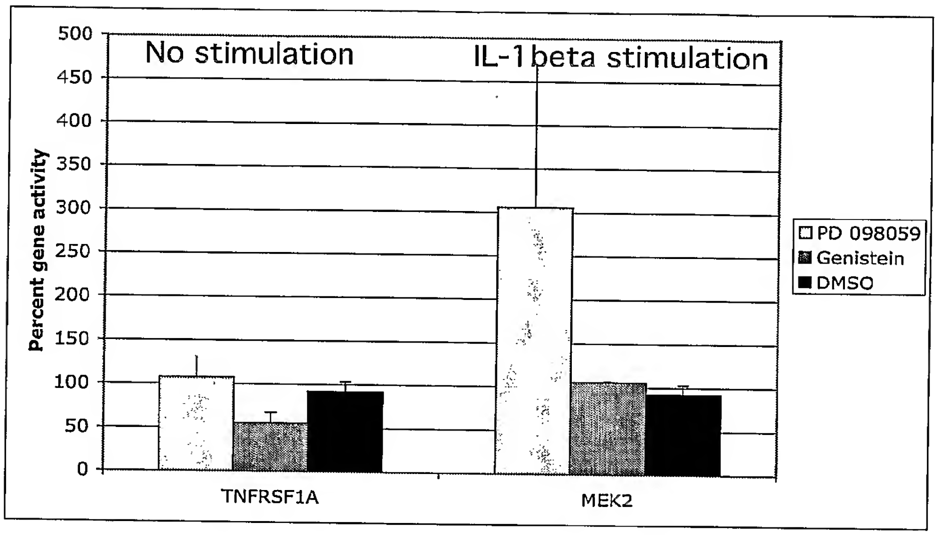
No stimulation
IL-1beta stimulation
Percent gene activity
500
450
400
350
300
250
200
150
100
50
0
TNFRSF1A
MEK2
PD 098059
Genistein
DMSO

Figure 4